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# A western blot assay to measure cyclin dependent kinase activity in cells or in vitro without the use of radioisotopes

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ABSTRACT

**We developed a quantitative method to measure the activity of cyclin-dependent kinases (Cdks) by western blotting, without radioisotopes. We prepared a recombinant protein substrate based upon the natural Cdk1 substrate, PP1Cα. By combining this substrate in a western blot method using fluorochrome based antibodies and phospho-imager analysis, we measured the $K_m$ of ATP binding to Cdk1 to be 3.5 μM. We then measured Cdk1 activity in cell extracts from interphase or mitotic cells, and demonstrated that previously identified Cdk inhibitors could be detected by this assay. Our data show that we have a safe, reliable assay to identify Cdk1 inhibitors and measure Cdk1 activity.**


## 1. Introduction

The measure of cyclin dependent kinase (Cdk) activity is a convenient means to identify cell cycle phases by using a biochemical approach. Cyclin dependent kinase 1 (Cdk1)/cyclin B1 complex is the key enzyme that drives mitosis; its protein subunits and role are conserved in eukaryotic species. It is a complex composed of a catalytic subunit (Cdk1) and one regulatory subunit cyclin B1, which is expressed in G2 and early M-phases of the cell cycle [1]. The activation of Cdk1/cyclin B1 complex is regulated by several different steps. Initially, the Cdk1/cyclin B1 complex is held inactive by phosphorylation of the ATP binding site. This permits the production of the complex, prior to its activation at the G2/M-phase transition through positive feedback mediated dephosphorylation. Exit from M-phase is coordinated by ubiquitin-dependent degradation of cyclin B1 [2]. When active, Cdk1 phosphorylates hundreds of different protein substrates [3], which results in cellular events such as morphology change, chromosome condensation, nuclear envelope breakdown, and segregation of condensed chromosomes. There are at least eleven other Cdk related proteins in cells, some, such as cyclin dependent kinase 2 (Cdk2), have phosphorylation activity that overlaps with that of Cdk1 [3]. Much of our understanding about cell proliferation in health and disease has been guided by measuring Cdk1 activity in cells.

Potential Cdk substrates can be identified by a canonical consensus sequence that includes a serine or threonine N-terminal to a proline with a basic amino acid such as a lysine or arginine within a −3 to +3 relative to the phosphoacceptor amino acid [4]. This sequence shares similarities to the phosphoacceptor sequences in substrates of the MAP kinase family, therefore biological evidence is required to establish protein kinase–substrate relationships. A Cdk consensus sequence is present in the human protein phosphatase PP1Cα at T320 [5,6]. Cdk1/cyclin B1 is reported to phosphorylate PP1Cα during mitosis, as demonstrated by western blotting with antibodies that distinguish phospho-T320 PP1Cα from non-phosphorylated T320 PP1Cα [7]. With the knowledge of the Cdk phosphorylation consensus sequence and by acquiring specific antibodies, it is possible to design specific assays to measure Cdk activity.

Current methods to measure Cdk1 activity in vitro use the histone H1 assay. In this technique, cell extracts or purified Cdk1 are combined with a buffer containing γ[$^{32}$P] ATP and incubated with histone H1 [8]. Phosphorylated histone H1 is then resolved by

*Abbreviations:* Cdk1, cyclin dependent kinase 1; Cdk2, cyclin dependent kinase 2; CPT, camptothecin; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EGTA, ethyleneglycol-bis (beta-aminoethylether)- *N,N′*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IPTG, isopropyl β-D-1-thiogalactopyranoside; PBS, phosphate-buffered saline

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SDS–PAGE and processed for autoradiography or it is collected on a solid support and counted by a detector. The amount of $P^{32}$ incorporated onto histone H1 is a measure of the activity of Cdk1 [8]. Although this method is widely used, there is a growing problem in handling radio-isotopes in research laboratories because of the dangers of exposure to ionizing radiation, requirements for specialized training, and handling of radioactive waste. These drawbacks motivated us to develop non-radioactive method of measuring Cdk1 activity. Other methods to measure Cdk1 activity include the use of a FRET biosensor that is composed of fluorescently labeled Cdk1 protein substrate and its binding partner [9]. The FRET system enables one to measure Cdk1 activity in live, individual cells, rather than a cell extract, as described here.

We have previously reported that we could measure Cdk1 activity in human cells undergoing mitosis with damaged DNA, a process known as checkpoint adaptation [10]. Since checkpoint adaptation had not been described in camptothecin (CPT) treated cells, a biochemical measure of Cdk1 in addition to morphological changes such as cell rounding was required to confirm mitosis [11]. In this paper, we describe the parameters of a method to measure Cdk activity using a recombinant protein substrate based upon human PP1Cα sequence and specific antibodies. We demonstrate that this assay is quantitative and that it can be used to detect Cdk small molecule inhibitors or Cdk1 activity in extracts prepared from human cells. We propose our assay as an alternative Cdk1 kinase assay to avoid the inconveniences and dangers of handling radio-isotopes.

## 2. Materials and methods

### 2.1. Cell culture

The human cell line HT-29 was obtained from the American Type Culture Collection. HT-29 cells were maintained in RPMI 1640 (Life Technologies) medium supplemented with 10% decomplemented fetal calf serum (PAA Laboratories, Etobicoke, Ontario), 2 mM L-glutamine (Invitrogen). The human cell line MCF7 was a gift from Dr. O. Kovalchuk. MCF7 cells were maintained in Advanced MEM (Life Technologies) media supplemented with 10% decomplemented fetal calf serum and 2 mM L-glutamine (Invitrogen). Cells were grown at 37 °C in 5% $CO_2$ and media were changed every second or third day. The compounds camptothecin (Sigma) and nocodazole (Sigma) were used at concentrations of 25 nM and 200 ng/mL, respectively.

### 2.2. Flow cytometry

Total cultures were collected by trypsinization. Cells were washed in phosphate-buffered saline (PBS) and fixed in 90% ethanol (−20 °C) for at least 24 h. For analysis, samples were incubated for 20 min in wash buffer with 0.02 mg/mL propidium iodide (Invitrogen) and 0.2 mg/mL RNAse A (Sigma), and analyzed by a FACS Canto II flow cytometer (BD Biosciences) using BD FACSDiva software. Gating was set using control samples. Experiments were repeated at least three times.

### 2.3. Extract preparation

Cells were resuspended in extraction buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.4, 50 mM NaF, 10 mM EGTA (ethyleneglycol-bis (beta-aminoethylether)- *N,N′*-tetraacetic acid), 50 mM β-glycerophosphate, 1 mM ATP, 1 mM (DTT) dithiothreitol, 1% Triton X-100, 10 μg/mL RNAse A, 0.4 U/mL DNAse I, with Roche protease inhibitors cocktail) at a concentration of 20,000 cells per μL, on ice for 30 min [12]. After five passages through a 26 g needle at 4 °C, the suspension was centrifuged at 10,000×*g* for 10 min at 4 °C. Extracts were used either for electrophoresis after being boiled for 5 min in presence of 2× SDS sample buffer for measurement of Cdk/cyclin activity.

### 2.4. Preparation of recombinant GST-PP1C-S

BL21 bacteria transformed with the pGEX-PP1Cα His plasmid were grown on a LB ampicillin (LB + AMP) plate at 37 °C. Single colonies were selected after 24 h and inoculated with LB + AMP liquid media. Tubes were placed on a shake tray for 2 h at 37 °C. When the culture was slightly turbid, it was transferred into 500 mL of LB + AMP liquid media. The optical density was monitored and the expression was induced at $OD_{600} = 0.6$, with 0.4 mM (IPTG) isopropyl β-D-1-thiogalactopyranoside. Samples were taken at set times until induction was stopped after 5 h. Samples were combined with 2× SDS reducing buffer and analyzed by SDS–PAGE. The remainder of the culture was centrifuged at 10,000×*g* for 20 min, the supernatant was removed, and the pellet was stored at −80 °C. Bacteria were thawed and pellet was re-suspended in lysis buffer (50 mM phosphate buffer pH 6.8, 1 mM EDTA, 100 mM NaCl, protease inhibitor (1/10 mL) at a ratio of 1 mL per g. Lysozyme solution (50 mg/mL Sigma L7651 in 10 mM phosphate buffer (pH 6.8), 1 mM EDTA) was then added at ratio of 50 μL/10 mL and incubated for 10 min. The extract was sonicated at 50% for 10 min and then centrifuged at 10,000×*g*. The supernatant was removed and the pellet was re-suspended with fresh lysis buffer and stored on ice. Recombinant GST-PP1C-S was purified by glutathione affinity chromatography using glutathione coated beads. The extract was passed through the column, which was washed three times with 10 mM phosphate buffer. Elution buffer (10 mM reduced glutathione and 50 mM Tris (pH 7.9)) was used to collect GST-PP1C-S. 100 μL samples of the flow through, phosphate buffer washes, and elutions were collected and analyzed by SDS–PAGE. GST-PP1C-S was dialyzed with 50 mM Tris (pH 7.9) for 6 h (final volume 9.5 mL). GST-PP1C-S was analyzed on a protein chip (On-Chip-Electrophoresis; Agilent Technologies) with a Bioanalyzer 2100 and estimated to be greater than 95% pure. GST-PP1C-S was stored at −80 °C until use.

### 2.5. Cdk1 kinase assay

Cdk1 phosphorylation reactions (20 μL total volume) were as follows: 10 μL of 2× Cdk1 phospho assay buffer (50 mM β-glycerophosphate pH 7.4, 10 mM $MgCl_2$, 10 mM NaF, 1 mM DDT) with 200 μM ATP (or as needed) and 5 μL of either 80 ng/μL GST or GST-PP1C-S. Reactions were initiated by adding either 5 μL of Cdk1/cyclin B1 (Millipore, 14–450) at a concentration of 8.6 ng/μL unless otherwise stated, Cdk2/cyclin A (Millipore, 14–448) as indicated or 5 μL of whole cell extracts diluted in lysis buffer (50 mM HEPES, pH 7.4; 1% Triton X-100; 50 mM NaF; 50 mM β-glycerophosphate; 10 mM EGTA; 1 mM ATP; DNase 1; 10 μg/mL RNase A; and 1 mM DDT) to 100 cells/μL. Final reactions contained 400 ng of either GST or GST-PP1C-S, and either 43.2 ng of Cdk1/cyclin B1 or an extract of the equivalent of 500 cells in a 20 μL volume. Reactions were incubated for 15 min at 30 °C. Reactions were stopped by adding an equal volume of 2× SDS sample buffer and heating to 95 °C for 5 min. All kinase reactions were performed at least three times. All compounds including those used in assays in vitro, CR8, and meriolins (ManRos Therapeutics, Roscoff, France), were dissolved in dimethyl sulfoxide (DMSO) to a concentration of 10 mM and stored at −20 °C until use.

### 2.6. Electrophoresis and western blotting

Reaction mixtures were separated in duplicate on a 12% SDS gel with a 4% stacking gel at 200 volts. Proteins were transferred to nitrocellulose with a semi-dry electroblotter system (BioRad) for 30 min at 25 volts. Subsequently, the membrane was blocked with either 5% low fat milk in Tris buffered saline TBS-0.1% Tween-20 (TBST) or 2% BSA in TBS-0.1% Tween-20 (TBS-T), and incubated overnight with the indicated primary antibody as follows: anti-phospho-PP1 alpha (Thr320) monoclonal antibody (Ab62334, Abcam, 1:300,000), or anti-phospho-PP1 alpha (Thr320) polyclonal antibody (2581S, Cell Signalling polyclonal, 1:1000) or anti-GST (G7781, Sigma–Aldrich, 1:20,000). After washing, the membrane was incubated with Alexa Fluor 488-coupled anti-mouse or anti-rabbit antibodies (A11059, A11008, Invitrogen, 1:400) or alkaline phosphatase conjugated anti-rabbit antibody (AP132A, Millipore, 1:2500). Western blots were performed at least three times.

### 2.7. Data analysis

Membranes were scanned with a Typhoon Trio Imager (Emission Filter: 526SP; Excitation: Blue 488; Voltage: 450–480 volts). Signals were analyzed using ImageQuant software. Lanes were manually drawn and the signal area was selected. A background section of similar dimensions to the signal detection box was then manually drawn and subtracted. The signal volume in positive pixels was determined based on a medium scan of 100 microns. Each signal was measured at least twice and the mean signal volume in fluorescent units was plotted using Excel software. Signals generated from GST membranes were used to standardize GST-PP1C-S signals. ATP-$K_m$ values were calculated from three independent experiments using the Lineweaver–Burk method and analyzed with Excel software to determine a best fit curve.

## 3. Results

The phosphorylation of human protein phosphatase 1Cα (PP1-Cα) on threonine 320 (T320) by Cdk1 has been previously described [7]. To confirm that this event occurs in other human cells, we cultivated the human cell colon carcinoma cell line HT-29 and tested it for the presence of phosphorylated T320 by western blotting. Cells were either not-treated, treated with 25 nM camptothecin, or with 200 ng/mL nocodazole, and analyzed at 24 h by flow cytometry to measure DNA content (Fig. 1A). As expected, the not-treated cells were predominantly in G1 with a G2/M-phase fraction, whereas the camptothecin treated cells were predominantly in S-phase and G2, and the nocodazole treated cells were in mitosis. We prepared extracts from these cultures and analyzed them by western blotting with anti-phosphoT320 PP1-Cα, or anti-phosphoY15 Cdk1, or anti-actin antibodies. The anti-phosphoT320 PP1-Cα signal was strongest in extracts prepared from nocodazole treated cells, which contained the mitotic cells (Fig. 1B). This sample contained the weakest anti-phosphoY15 Cdk1 signal, confirming that the cells were in mitosis. By contrast the camptothecin treated cells had a strong anti-phosphoY15 Cdk1 signal. The anti-actin western blot confirmed that equal amounts of protein were present in each extract. These data confirmed that the phospho-T320 PP1-Cα antibodies detect phosphoT320 PP1-Cα in HT-29 cell extracts prepared at the time when Cdk1 is active.

We recovered the human sequence of PP1-Cα from public data bases (Supplementary Fig. S1) and designed cDNA sequences encoding amino acids 316–324 that flanked the phosphosite T320 (Fig. 2A). We also incorporated six histidines to the C-terminus of the recombinant protein as a second epitope for anti-histidine antibodies. These sequences were subcloned into a pGEX plasmid to encode GST with a C-terminus that has the nine amino acids from PP1-Cα followed by six histidines. The recombinant protein, named GST-PP1C-S (S for substrate), is composed of 242 amino acids with a molecular weight of 28.5 kD (Fig. 2A).

We produced the recombinant protein in *Escherichia coli* that harboured the plasmid pGEX-GST-PP1C-S and purified GST-PP1C-S or GST proteins by glutathione bead affinity chromatography (Supplementary Fig. S1). We compared GST-PP1C-S, GST or proteins from total human cell extracts by western blot analysis with anti-GST antibodies, or anti-6 histidine antibodies or anti-pT320 PP1-Cα antibodies (Fig. 2B). The GST-PP1C-S was recognized by the GST and 6 histidine antibodies (Fig. 2B, Lanes 3 and 4). In its non-phosphorylated form as purified from *E. coli*, it was not recognized by anti-pT320 PP1-Cα antibodies, although phosphorylated PP1-Cα from a mitotic cell extract was recognized. These data

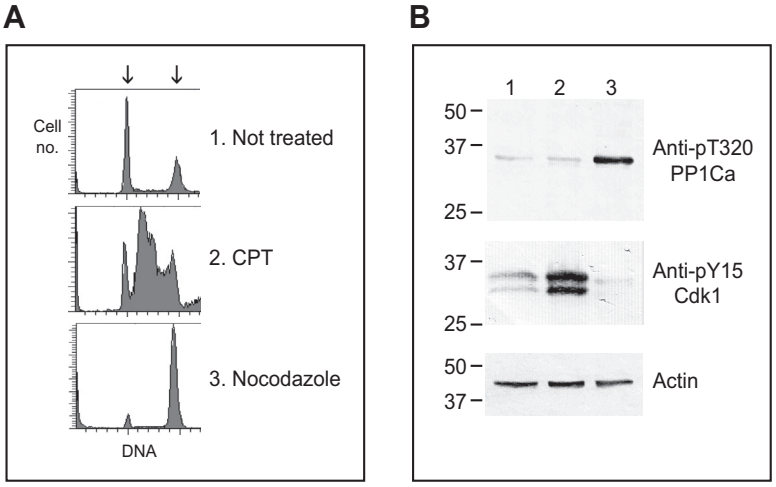


**Fig. 1.** Protein phosphatase 1Ca is phosphorylated on T320 during mitosis. (A) Human colon carcinoma cells (HT-29) were either not treated (top panel) or treated with 25 nM CPT for 24 h (middle panel) or treated with nocodazole for 24 h (bottom panel). DNA content was determined by propidium iodide staining and flow cytometry. The arrows indicate the 2N and 4N DNA positions. (B) HT-29 cells were either not treated (lane 1) or treated with 25 nM CPT for 24 h (lane 2) or treated with nocodazole for 16 h (lane 3). Extracts were prepared and analyzed by western blotting with either anti-pT320 PP1-Cα, or anti-pY15 Cdk1 or anti-actin antibodies. Positions of molecular weight markers in kDa are shown on the left.

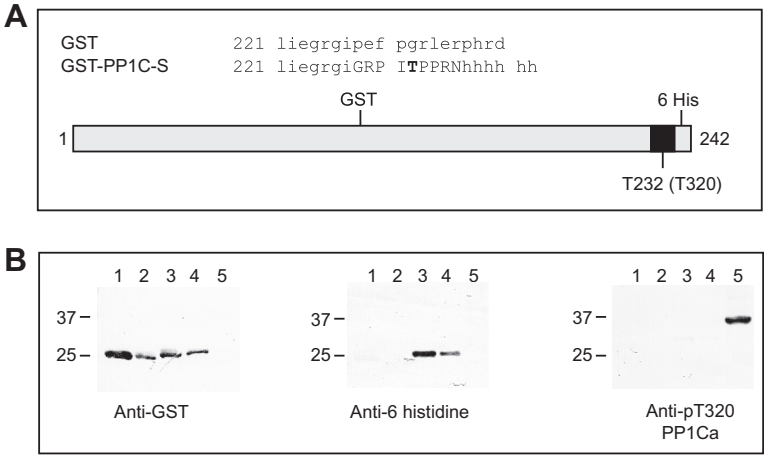


**Fig. 2.** Amino acid sequence and composition of the recombinant protein GST-PP1C-S. (A) The amino acid sequences, starting from amino acid 221, from GST (top row) or GST-PP1C-S (bottom row) are provided. T232, which is equivalent to T320 of human PP1-Cα is marked in bold and the amino acids derived from human PP1-Cα are shown in capital letters. Note that T232 is the only phosphorylatable amino acid that is present in GST-PP1C-S but not in GST. A schematic drawing of GST-PP1C-S that highlights the key motifs is shown. (B) Two concentrations of GST (lanes 1; 100 ng, 2; 50 ng) and two concentrations of GST-PP1C-S (lanes 3; 100 ng, 4; 50 ng) and a total cell extract from nocodazole treated HT-29 cells (lane 5) were analyzed by western blotting and developed with an anti-GST antibody or with an anti-6 histidine antibody, or with an anti-pT320 PP1-Cα antibody. Positions of molecular weight markers in kDa are shown on the left.

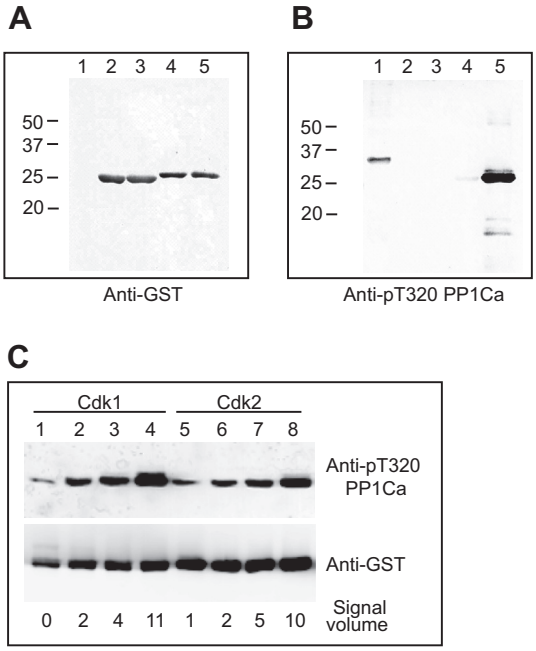


**Fig. 3.** Phosphorylation of GST-PP1C-S by Cdk1 and Cdk2. (A) GST (lanes 2, 3) or GST-PP1C-S (lanes 4, 5) were either incubated in kinase buffer (lanes 2, 4) or kinase buffer and Cdk1/cyclin B1 (lanes 3, 5). A total cell extract from nocodazole treated HT-29 cells was prepared in parallel (lane 1). Samples were analyzed by western blotting and developed with an anti-GST antibody. Positions of molecular weight markers in kDa are shown on the left. (B) Samples, as described in A, were analyzed by western blotting and developed with an anti-pT320 PP1-Cα antibody. (C) Equal units of Cdk1/cyclin B1 or Cdk2/cyclin A were incubated with GST-PP1C-S (Lanes 1 and 5, 0 mU; lanes 2 and 6, 5 mU; lanes 3 and 7, 14 mU; lanes 4 and 8, 40 mU). Samples containing 100 ng of substrate were analyzed by western blotting and developed with anti-GST or anti-pT320 PP1Ca antibodies. The signal volumes were measured by phospho-imager analysis and are shown below each lane.

showed that we could produce and purify GST-PP1C-S, and that in its native state, it did not interact with the anti-T320 PP1-Cα antibody.

We then tested if recombinant GST-PP1C-S could be phosphorylated by Cdk1, so that it would be recognized by anti-pT320 PP1-Cα antibodies. Purified GST-PP1C-S was incubated with purified Cdk1 in a protein kinase buffer that contained ATP (Fig. 3). In control tests, the same procedure was followed but without ATP. The reactions were stopped by the addition of SDS-sample buffer and analyzed by western blotting with either anti-GST antibodies or with anti-pT320 PP1-Cα antibodies. GST and GST-PP1C-S were detected by an anti-GST western blot (Fig. 3A). By contrast, GST-PP1C-S was readily detected by the anti-pT320 PP1-Cα antibodies when GST-PP1C-S was incubated with Cdk1 (Fig. 3B lane 5), whereas GST protein was not detected. The only phosphate acceptor amino acid that is present in GST-PP1C-S and not present in GST, is threonine 232 (Fig. 2A). When GST-PP1C-S is not phosphorylated by Cdk1, it is not readily detected by the anti-phosphoT320 PP1Ca antibody (Supplementary Fig. S2A). We then compared GST-PP1C-S phosphorylation by similar units of Cdk1/cyclin B1 or Cdk2/cyclin A, and found that these closely related kinases phosphorylated the substrate to similar levels (Fig. 3C). We noted, however, that the specific activity of Cdk2 in these experiments was five times lower than that of Cdk1, therefore, to achieve similar levels of GST-PP1C-S phosphorylation, we used five times more Cdk2/cyclin A protein. These data demonstrate that GST-PP1C-S can be phosphorylated by the two major Cdk family members.

To explore the kinetic parameters of GST-PP1C-S phosphorylation by Cdk1, we varied the concentration of ATP while maintaining protein substrate concentration (Supplementary Fig. S3). We found that 50 μM of ATP was within the saturation region of the reaction kinetics, a value that we use in standard assay conditions. Michalis–Menton analysis of the signal volumes from western blotting of GST-PP1C-S phosphorylation revealed a $K_m$ for ATP to be 3.5 μM ($N = 3$). These data suggested that the western blot detection system could be used to measure Cdk1 activity quantitatively.

With a robust assay to measure Cdk activity in vitro, we then asked if the western blot method could be used to detect known small molecule Cdk inhibitors. We set up the assay under standard conditions, although we reduced the ATP concentration to 3 μM, which is at $K_m$. Prior to starting the assay we added either meriolin, a potent Cdk inhibitor, at three different concentrations or we added methyl-meriolin, an inactive analogue from the same chemical family [13]. Meriolin strongly inhibited Cdk1 at 10 and 1 μM concentrations, as previously reported, but now detected by the

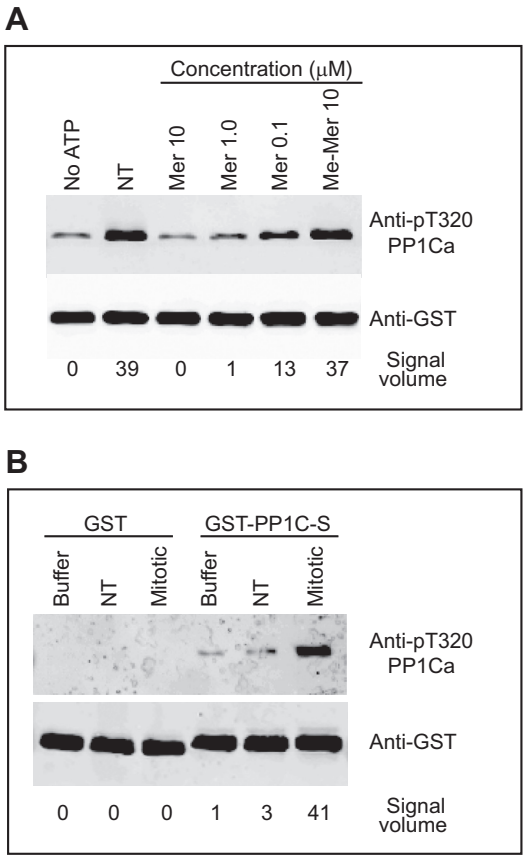


**Fig. 4.** The phosphorylation of GST-PP1C-S can be inhibited by Cdk small molecule inhibitors and GST-PP1C-S is phosphorylated by mitotic extracts. (A) GST-PP1C-S was incubated in kinase buffer without ATP (lane 1) or with 3 μM ATP (lanes 2–6). In addition, samples were incubated with either 10 μM meriolin (lane 3), or 1 μM meriolin (lane 4), or 0.1 μM meriolin (lane 5) or 10 μM methyl-meriolin (lane 6). Samples containing 100 ng of substrate were analyzed by western blotting and developed with anti-pT320 PP1-Cα (top row) or anti-GST antibodies (bottom row). The signal volumes of the anti-pT320 PP1-Cα western blot were estimated by phospho-imager analysis. Inhibitor testing experiments were performed three times and a representative result is shown. (B) GST (lanes 1–3) or GST-PP1C-S (lanes 4–6) were either incubated in kinase buffer (lanes 1, 4) or with cell extracts prepared from not-treated HT-29 cells (lanes 2, 5) or extracts prepared from nocodazole treated HT-29 cells (lanes 3, 6). Equal amounts of cell extracts were used in each sample. Samples were analyzed by western blotting and developed with anti-pT320 PP1-Cα (top row) or anti-GST antibodies (bottom row). The signal volumes of the anti-pT320 PP1-Cα western blot were estimated by phospho-imager analysis. Experiments were performed three times and a representative result is shown.

western blotting assay (Fig. 4A). Methyl-meriolin did not prevent the phosphorylation of GST-PP1C-S by Cdk1. In related experiments, we found that other Cdk inhibitors, including CR8 [14] or meriolin active analogues, inhibited GST-PP1C-S phosphorylation (Supplementary Fig. S2B). These data confirmed that the western blot assay can be used to detect or to verify the activity of Cdk1 inhibitors.

Knowing that we could reliably and quantitatively detect Cdk1 activity in vitro, we tested if we could detect Cdk1 activity present in total extracts of human cancer cells. To obtain mitotic cell extracts, HT-29 cells were first treated with nocodazole as described in Fig. 1. Non-treated cells were treated with the equivalent amount of the solvent DMSO. Extracts were prepared, incubated with either GST or with GST-PP1C-S, and analyzed by western blotting. In parallel, the assay was performed using only extraction buffer. The GST only substrate did not produce any signals by blotting with the anti-pT320-PP1-Cα antibody (Fig. 4B). By contrast, a relatively strong signal was detected when the GST-PP1C-S substrate was incubated with mitotic extracts. The signal was approximately 10 times more intense than that of non-treated cell extracts and 40 times more intense than a buffer only (background) signal.

Although we demonstrated that GST-PP1C-S is phosphorylated by Cdk1 and in cells extracts when Cdk1 is active, it remained possible that the TP motif might be phosphorylated by the Erk1/2 kinases, which also recognize TP or SP amino acids. To test this, we prepared extracts from MCF7 cells treated with etoposide, a topoisomerase II inhibitor that induces Erk1/2 activation [15] (Fig. 5A). Despite the increase in Erk1/2 activation, we did not detect GST-PP1C-S phosphorylation when comparing not-treated and etoposide treated extracts. By contrast, HT-29 cells did not demonstrate Erk1/2 activation when treated with nocodazole to produce mitotic cells, (Fig. 5A). Under the conditions used here, only the mitotic sample displayed GST-PP1C-S activity in HT-29 cells, even though similar levels of Erk1/2 phosphorylation were present in the not-treated and mitotic cells. These data support the conclusion that the assay can be used to measure Cdk activity from extracts prepared from cells in interphase or in mitosis.

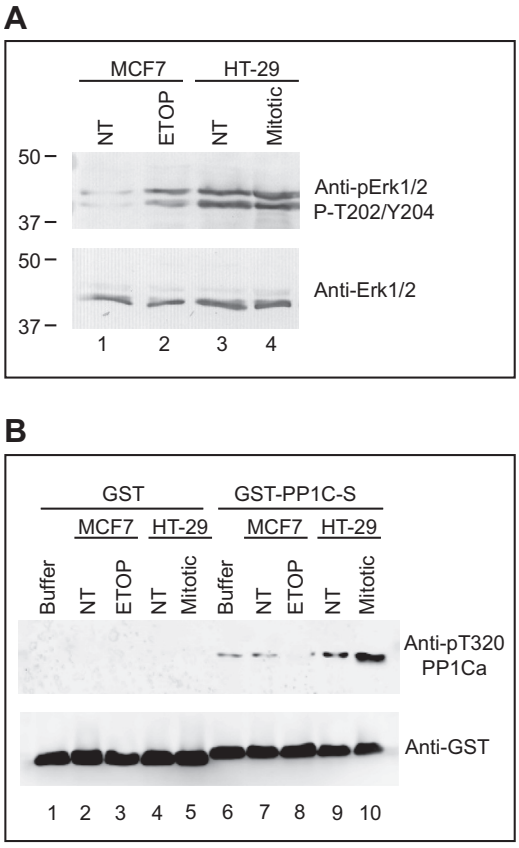


**Fig. 5.** GST-PP1C-S is not phosphorylated in cell extracts containing activated Erk1/2. (A) MCF7 cells were either not treated (lane 1) or treated with 20 μM etoposide for 4 h (lane 2). In parallel, HT-29 cells were either not treated (lane 3) or treated with nocodazole for 24 h (lane 4). Extracts were prepared and analyzed by western blotting with either anti-pERK1/2 P-T202/Y204 or anti-ERK1/2 antibodies. Equal amounts of cell extract were loaded in each lane. Positions of molecular weight markers in kDa are shown on the left. (B) GST (lanes 1–5) or GST-PP1C-S (lanes 6–10) were either incubated in kinase buffer (lanes 1, 6) or with cell extracts prepared from not-treated MCF7 cells (lanes 2, 7) or etoposide treated MCF7 cells (lanes 3, 8) or not-treated HT-29 cells (lanes 4, 9) or nocodazole treated HT-29 cells (lanes 5, 10). Equal amounts of cell extracts were used in each sample. Samples containing 100 ng of substrate were analyzed by western blotting and developed with anti-pT320 PP1-Cα (top row) or anti-GST antibodies (bottom row). The experiment was repeated twice and a representative result is shown.

## 4. Discussion

We developed a new recombinant protein substrate and adapted the technique of western blotting to measure Cdk1 activity quantitatively in biochemical assays and cell extracts. In designing a Cdk1/cyclin B1 substrate, we looked for one that was phosphorylated uniquely in mitosis. We used the human enzyme PP1-Cα as described by Kwon et al., which is phosphorylated on T320 by Cdk1 during mitosis [7]. We validated these results prior to launching a new Cdk1 assay (Fig. 1). We then prepared bacterial expression plasmids using GST and 6-histidine tag sequences in-frame with the published a nine amino acid sequence that contained the PP1-Cα phosphorylation site. The GST tag has three roles in this assay: it is used to purification the substrate, to quantify signal strength in assays, and it makes the recombinant substrate large enough so that phosphorylation can be evaluated by SDS–PAGE. We routinely include a 6 histidine sequence on the C-termini of recombinant proteins to aid in purification, if required, and to provide a convenient epitope to confirm an intact C-terminus (Fig. 2).

We tested two p-T320 PP1-Cα antibodies against GST-PP1C-S and GST to find the antibody and substrate concentrations under which they were unlikely to interact with each other (Supplementary Fig. S4). For convenience, we chose to work with a monoclonal antibody, which ensures that the antibody-substrate interaction does not change after stock renewal. We based our choice of secondary antibody upon excitation/emission spectra available with our model of the phospho-imager, and upon signal strength of several different types of fluorochromes available within those detection limits. Detailed analyses of fluorochromes and detection have been described elsewhere [16]; we found empirically that the Alexafluor 488 provided a robust intense signal that contributed to a 20-fold linear range of detection (data not shown).

A phospho-imager detection system is ideal because of its ease of use, rapid detection times and capacity to collect signals over a large range of intensities. We compared signal strength of phosphorylated GST-PP1C-S acquired from a range of ATP concentrations. The data were analyzed by the Michalis–Menton method and a $K_m$ of 3.5 μM was calculated, which was lower that the $K_m$ identified when using histone H1 substrates [17]. To calibrate signals between experiments we would suggest including known phosphorylated GST-PP1C-S samples on successive SDS polyacrylamide gels. We would only compare signals from within the same western blot analysis. For our standard assays, we routinely used 100 μM ATP substrate, which is approximately 30 times above the $K_m$ and within the saturation region of the signal volume (Supplementary Fig. S3).

In assays to detect Cdk inhibitors, we used the ATP-$K_m$ concentration 3 μM ATP, a common practise in protein kinase inhibition assays [18]. Inhibition of Cdk1 by meriolin ($IC_{50}$ 0.01 uM) [19] or CR8 ($IC_{50}$ 0.4 uM) [14] were readily detected, whereas the inactive, methylated form of meriolin, did not inhibit Cdk1. Although other methods, including those using radiolabeled substrates are likely more suitable for routine or high-throughput screening [20], the western blot method is convenient for periodic analysis of a small number of inhibitors.

The substrate can be recognized by Cdk2/cyclin A and Cdk1/cyclin B in tests in vitro. In cell based assays, we set the conditions to detect mitotic Cdk1 activity, which likely do not detect other Cdks, including Cdk2 complexes. These conditions have been previously used to detect Cdk1 in cell extracts by histone H1 kinase assays [12]. We excluded the possibility that we were measuring Erk1/2 kinase, which have some substrate overlap with Cdks. Under conditions of Erk1/2 activation, we were not able to detect phosphorylation of the substrate. We had previously reported that Cdk1 activity was high in cells that had undergone checkpoint adaptation [10]. Here, we provide experimental detail to validate the method and demonstrate that the assay detects Cdk1 activity in mitotic cells and that this activity is approximately 10-fold higher than that of an exponentially growing, not-treated culture (Fig. 4), as has been previously reported by other methods [12]. To maintain signals within the linear range of the assay, we found that we could dilute extracts to an equivalent of 500 cells, suggesting that the detection system is sensitive and can be applied to most standard culture conditions. The ease of preparing the substrate and the availability of a monoclonal antibody make this assay suitable for either routine or periodic use by a common method of western blotting.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.08.003.

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